

Adaptation of Iron Homeostasis Pathways by a *Pseudomonas aeruginosa* Pyoverdine Mutant in the Cystic Fibrosis Lung

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Cystic fibrosis (CF) patients suffer from chronic bacterial lung infections, most notably by *Pseudomonas aeruginosa*, which persists for decades in the lungs and undergoes extensive evolution. *P. aeruginosa* requires iron for virulence and uses the fluorescent siderophore pyoverdine to scavenge and solubilize ferric iron during acute infections. Pyoverdine mutants accumulate in the lungs of some CF patients, however, suggesting that the heme and ferrous iron acquisition pathways of *P. aeruginosa* are more important in this environment. Here, we sought to determine how evolution of *P. aeruginosa* in the CF lung affects iron acquisition and regulatory pathways through the use of longitudinal CF isolates. These analyses demonstrated a significant reduction of siderophore production during the course of CF lung infection in nearly all strains tested. Mass spectrometry analysis of one of these strains showed that the later CF isolate has streamlined the metabolic flux of extracellular heme through the HemO heme oxygenase, resulting in more-efficient heme utilization. Moreover, gene expression analysis shows that iron regulation via the PrrF small RNAs (sRNAs) is enhanced in the later CF isolate. Finally, analysis of *P. aeruginosa* gene expression in the lungs of various CF patients demonstrates that both PrrF and HemO are consistently expressed in the CF lung environment. Combined, these results suggest that heme is a critical source of iron during prolonged infection of the CF lung and that changes in iron and heme regulatory pathways play a crucial role in adaptation of *P. aeruginosa* to this ever-changing host environment.

Cystic fibrosis (CF) is a heritable disease characterized by the accumulation of thick, dehydrated mucus in the lungs, making patients prone to infections by numerous bacteria (1, 2). *Pseudomonas aeruginosa*, a Gram-negative opportunistic pathogen, infects approximately 80% of adult CF patients by early adulthood (2, 3), with initial colonization occurring in the presence of a diverse CF lung microbiome (1). *P. aeruginosa* eventually becomes the predominant resident of the CF lung, where it persists for decades as a chronic infection (2). During this period, the physiology of the CF lung changes dramatically due to chronic infection and inflammation, resulting in considerable evolution of infecting *P. aeruginosa* strains. Most notable is the eventual conversion of most CF isolates of *P. aeruginosa* to a mucoid phenotype, characterized by increased polysaccharide production (4, 5) and antibiotic resistance (6–9) and decreased production of factors required for acute infections (10–16).

Iron is required for *P. aeruginosa* virulence (17–22) but is sequestered from microbial pathogens in the human host (23, 24). *P. aeruginosa* overcomes iron limitation during infection through a variety of mechanisms, including the synthesis and secretion of two siderophores, pyoverdine (25) and pyochelin (26). These molecules scavenge ferric iron from host proteins during infection and are thus required for pathogenesis in a number of virulence models (17–19, 22). Upon binding to the FpvA outer membrane receptor, pyoverdine additionally activates gene expression via the PvdS sigma factor, inducing production of an iron-regulated protease, exotoxin A, and pyoverdine biosynthesis proteins (27–29). As such, the requirement of pyoverdine in acute infections is due to both high-affinity iron acquisition and regulation of virulence gene expression. Pyoverdine-mediated uptake of ferric iron may not be as important during chronic CF lung infections, however, given the finding that disease progression is associated with decreased oxygen levels (30). In line with this hypothesis, pyoverdine

is present in the sputum of some but not all CF patients (31), and in one study, one-third of *P. aeruginosa* strains isolated from CF patients had lost the ability to produce pyoverdine (32). Additionally, expression of the genes for pyoverdine biosynthesis by *P. aeruginosa* growing in the CF lung is variable (33). Moreover, recent genome sequencing analysis of numerous CF isolates demonstrated the highest level of genetic diversity among infecting *P. aeruginosa* strains to be localized to the pyoverdine synthesis and uptake genes (34).

Based on the studies cited above, it is hypothesized that *P. aeruginosa* adapts to use ferrous iron and heme uptake systems in place of siderophores during CF lung infection. In support of this idea, genes encoding the ferrous iron and heme uptake systems are consistently expressed by *P. aeruginosa* growing in the CF lung (33, 35). *P. aeruginosa* acquires ferrous iron via the Feo system, a G-protein-like transporter of ferrous iron (36–39), and mediates heme acquisition via at least two systems: Phu (*Pseudomonas* heme uptake) and Has (heme assimilation system) (40). Once internalized, heme is bound by the cytoplasmic heme chaperone PhuS and delivered to an iron-regulated heme oxygenase (*hemO*), which degrades heme to iron, CO, and biliverdin (BVIX) (41). *P. aeruginosa* is unique in that many strains encode a second, non-

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iron-regulated heme oxygenase, BphO (42). However, *in vitro* studies show that PhuS can deliver heme to HemO but not to BphO (43). Further, *P. aeruginosa* strain PAO1 almost exclusively degrades extracellularly provided heme with HemO, while BphO mediates degradation of endogenously produced heme (44). Combined, these studies demonstrated that *P. aeruginosa* HemO is likely the primary driver of iron acquisition from exogenous heme in strain PAO1.

While iron is required for growth and virulence of *P. aeruginosa*, surplus iron can also cause oxidative stress. The central mediator of iron homeostasis in *P. aeruginosa* is the ferric uptake regulator (Fur) protein, and several lines of evidence indicate the essentiality of *P. aeruginosa* Fur (45–47). In iron-replete environments, ferrated-Fur represses expression of genes for iron acquisition, preventing toxic iron accumulation in the cytosol (48). Fur also mediates positive regulation of numerous genes via repression of the PrrF small RNAs (sRNAs), which in turn negatively affect the expression of more than 50 genes encoding iron storage proteins, enzymes that protect against oxidative stress, and iron-containing respiratory enzymes (49, 50). Accordingly, PrrF regulation spares intracellular iron stores during growth in iron-limiting environments, while Fur repression of the PrrF sRNAs allows for functions that are favored when iron is more readily available. The *P. aeruginosa* PrrF sRNAs are encoded in tandem, allowing the expression of a heme-regulated sRNA named PrrH (51). We previously showed that PrrH mediates heme activation of *nirL* (51), encoding a putative activator of heme d_1 -dependent nitrite reductase (NIR) required for anaerobic respiration (52), suggesting that this novel sRNA regulates heme homeostasis.

Among the metabolic genes regulated by PrrF are those encoding the enzymes for degradation of anthranilate. Anthranilate is the substrate for PqsA, which carries out the first step in biosynthesis of multiple 2-alkyl-4-quinolones (AQ) (53). One of these AQs, the *Pseudomonas* quinolone signal (PQS), is a quorum-sensing molecule that regulates virulence gene expression (54, 55). The PrrF sRNAs spare anthranilate for PQS production by inhibiting the expression of the anthranilate degradation genes (50). Thus, the PrrF sRNAs are required for PQS production under low-iron growth conditions. Purified PQS can also stimulate pyoverdine biosynthesis via its ability to chelate and sequester extracellular ferric iron. As a result of this activity, purified PQS blocks the growth of *P. aeruginosa* siderophore mutants (56, 57). These findings have led to the proposal that PQS functions in extracellular iron entrapment to facilitate pyoverdine-mediated iron uptake by wild-type *P. aeruginosa*. Additionally, PqsA is required for lysis and iron acquisition from the Gram-positive bacterium *Staphylococcus aureus* (58), which inhabits the CF lung at earlier stages of disease (2). As such, pyoverdine, PrrF, and PQS may play interrelated roles in iron acquisition and regulation in *P. aeruginosa*, particularly in polymicrobial infections. However, it remains unknown how these different activities work together to mediate iron acquisition during chronic CF lung infections.

Pyoverdine clearly plays a central role in *P. aeruginosa* physiology and virulence, and loss of this important virulence factor during chronic CF infection is likely accompanied by significant changes in gene regulation and iron acquisition pathways. In this report, we show that pyoverdine production was reduced during CF lung infection in four of five longitudinally isolated strains of *P. aeruginosa*. In-depth analysis of one of these strains revealed considerable changes in iron acquisition and regulatory systems at

later stages of infection. Most notable were loss of PQS production, changes in PrrF-mediated iron regulation, and enhanced heme utilization. This report provides the first in-depth characterization of the long-term changes that occur in iron homeostasis during chronic infections of the CF lung by *P. aeruginosa*. Further, this work provides the basis for identification of novel iron acquisition and regulatory targets for future antimicrobial drug development directed toward chronic lung infections.

MATERIALS AND METHODS

Bacterial strains, genetic manipulations, and growth conditions. Longitudinal isolates from CF patients were isolated at hospitals in British Columbia (David Speert) as previously described (11) and are described in Table 1. Other bacterial strains and plasmids used in this study are listed in Table S1 in the supplemental material. Random amplified polymorphic DNA (RAPD) typing of longitudinal isolates was performed as previously described using primer 272 (AGCGGGCCAA) (59). The *pqsA* mutant was generated in our laboratory's PAO1 strain by allelic exchange (60) using the previously described $\Delta pqsA$ deletion construct (61). Brain heart infusion (BHI) media were used for routine culture of *P. aeruginosa*. Luria-Bertani (LB) medium was used for routine culture of *E. coli*. For expression and heme metabolism studies, strains were grown for 18 h at 37°C with aeration in Chelex-treated, dialyzed Trypticase soy broth (DTSB) or diluted from LB medium into M9 minimal media purchased from Teknova containing 2% glucose and grown for 4 h and then subcultured into fresh M9 media for an additional 8 h at 37°C (62). Ferric chloride was added to achieve a final concentration of 100 μ M or 200 μ M as indicated. Heme stocks were freshly prepared prior to use in 0.1 N NaOH–20 mM Tris and adjusted to pH 7.0 with HCl, and the stock concentration was determined by a pyridine hemochrome assay as previously described (63) using the following extinction coefficients: $\epsilon = 170.0 \text{ mM}^{-1} \text{ cm}^{-1}$ at $\lambda = 418 \text{ nm}$; $\epsilon = 17.5 \text{ mM}^{-1} \text{ cm}^{-1}$ at $\lambda = 525 \text{ nm}$; and $\epsilon = 34.5 \text{ mM}^{-1} \text{ cm}^{-1}$ at $\lambda = 555 \text{ nm}$. Heme was immediately diluted into media to the indicated concentration.

Detection of siderophores. Production of the pyoverdine chromophore was determined spectroscopically by reading the absorbance of culture supernatants at 410 nm (64). Total iron chelator production in DTSB and M9 culture supernatants was quantified by a chrome azurol S (CAS) assay as previously described (65). All readings were normalized to culture density as determined by the absorbance at 600 nm.

Real-time PCR. Real-time PCR quantitative PCR (qPCR) analysis of gene expression in broth cultures was carried out as previously described (51, 62, 66) using an Applied Biosystems StepOne Plus real-time PCR system (Life Technologies). Primers and probes are listed in Table S2 in the supplemental material. Relative amounts of cDNA were determined by the $\Delta\Delta C_T$ (threshold cycle) method or by use of a standard curve generated from serial dilutions of cDNA from strain PAO1 grown under low-iron conditions. Expression was normalized to the *omlA* cDNA detected in each sample. Sputum samples were collected under the approval of the New Zealand Health and Disability Ethics Committees (NYTY/10/12/106) and the Southern Tasmanian Health and Medical Research Ethics Committee (H9813). Individuals with CF and non-CF bronchiectasis attending Dunedin Hospital were recruited, and written informed consent was provided by all study participants. Sputum was expectorated into 20 ml of RNeasy (Qiagen). RNA was extracted, cDNA synthesized, and qPCR carried out as described previously (33), with transcript amounts being normalized to those of the *clpX* and *oprL* reference genes.

Detection of PQS. Bacteria were grown in DTSB for 18 h at 37°C, with and without 100 μ M FeCl_3 supplementation as indicated. Each culture was harvested and extracted with acidified ethyl acetate as described by Collier et al. (67). One-half of the resulting organic extract was transferred to a clean tube and evaporated to dryness. Samples were resuspended in 1:1 acidified ethyl acetate:acetonitrile and analyzed by thin-layer chromatography (TLC) (68).

TABLE 1 Summary of pyoverdine production by longitudinal isolates analyzed in this study

Isolate	Initial characterization				Siderophore production ^a			
	Date of collection (mo/day/yr)	Patient age	Pigmentation	Mucoid	DTSB		M9	
					CAS activity	Pyoverdine chromophore	CAS activity	Pyoverdine chromophore
JSRI-1	6/13/1989	8 yrs, 5 mos	Yellow-green	No	+	+	+	+
JSRI-2	5/13/1997	17 yrs, 6 mos	Green	Yes	—	—	—	—
JSRI-3	5/30/2003	23 yrs, 6 mos	Blue	Yes	N.D.	N.D.	N.D.	N.D.
DSAM-1	8/22/1983	11 yrs, 10 mos	Yellow	No	++	++	+	+
DSAM-2	4/4/1993	18 yrs, 2 mos	Yellow	No	+	—	—	—
DSAM-3	7/22/1997	22 yrs, 1 mos	Yellow	No	—	—	—	—
FCOR-1	7/23/1985	4 yrs, 2 mos	Green	No	++	+	+	+
FCOR-2	2/17/1993	12 yrs, 3 mos	Blue	Yes	+	+	—	—
FCOR-3	9/25/1999	18 yrs, 4 mos	Blue	Yes	—	—	+	+
LNAP-1	12/18/1982	2 yrs, 11 mos	Yellow	No	++	+	+	+
LNAP-2	4/21/1993	13 yrs, 3 mos	Yellow	No	++	++	—	—
LNAP-3	5/27/1999	19 yrs, 4 mos	Green-red	No	+	+	+	+
WTHO-1	11/25/1985	5 yrs, 6 mos	Blue-green	Yes	++	+	+	—
WTHO-2	4/19/1994	13 yrs, 11 mos	Yellow-green	Yes	++	+	+	+
WTHO-3'	10/2/1994	14 yrs, 5 mos	Red	No	—	—	—	—
WTHO-4'	9/21/1999	19 yrs, 4 mos	Red	No	+	+	+	+

^a Summary of siderophore production as shown in Fig. 2 and Fig. S4 in the supplemental material. The following symbols indicate pyoverdine production levels as follows: ++, significantly greater than PAO1; +, equivalent to PAO1; —, significantly less than PAO1; N.D., not determined. CAS reactivity was determined as described in Materials and Methods. Pyoverdine chromophore was detected in supernatants at 410 nm as described in Materials and Methods.

Isotopic heme labeling studies. Heme labeling studies were performed as previously described (66). Briefly, heme was prepared from unlabeled or labeled [4-¹³C]-δ-aminolevulinic acid (ALA) according to the method described by Rivera and Walker (69) and the yield calculated by the pyridine hemochrome assay (70). *P. aeruginosa* strains were grown in M9 media supplemented with the indicated concentrations of [¹²C]heme or [¹³C]heme. BVIX isomers were purified from the culture supernatants and separated and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Waters TQD triple-quadrupole mass spectrometer with Aquity H-class ultraperformance LC [UPLC]) as described previously (66). Fragmentation patterns of the parent ions at 583.21 ([¹²C]BVIX) and 591.21 ([¹³C]BVIX) were analyzed using multiple-reaction monitoring (MRM). The fragmentation patterns of the respective BVIX isomers are shown in Scheme S1 in the supplemental material.

RESULTS

Pyoverdine production is significantly reduced in longitudinal isolates from multiple CF patients. To determine the molecular basis of pyoverdine mutation in the CF lung, we first sought to identify natural pyoverdine biosynthesis mutants from a collection of longitudinal CF clinical isolates of *P. aeruginosa*. Among these strains are deidentified isolates from longitudinal studies of infants, children, and adults exhibiting both mucoid and nonmucoid phenotypes. From this collection, we chose 16 isolates with various levels of fluorescent pigmentation that were isolated from five different CF patients. A summary of these isolates, their pigmentation and mucoid characteristics, collection dates, and subject age at collection is provided in Table 1. Random amplified polymorphic DNA (RAPD) typing of these isolates demonstrated that those from the patients corresponding to designations JSRI, LNAP, DSAM, and FCOR were indeed clonal (Fig. 1A and B). As a comparison, other commonly used laboratory strains and non-longitudinal CF isolates showed distinct banding patterns (Fig. 1A). WTHO-1 and WTHO-2, which were isolated from a single patient earlier in infection (ages 5 and 13), were also related to one

another. However, WTHO-3' and WTHO-4', which were isolated from the same patient at later stages of infection (ages 14 and 19), were clones of a distinct *P. aeruginosa* strain (Fig. 1C). Most of the isolates grew at rates comparable to that of the wild-type PAO1 strain (see Fig. S1 and S2 in the supplemental material). However, the JSRI-3 isolate, which is a small-colony variant, did not grow well in most laboratory media, including Luria-Bertani (LB) broth (see Fig. S3 in the supplemental material). Therefore, this isolate was not amenable to further analysis in this study.

We next characterized siderophore production by each of these isolates in comparison with laboratory strain PAO1 and an isogenic $\Delta pvdA$ mutant, which lacks the enzyme for the first dedicated step in pyoverdine biosynthesis. Strains were grown in dialyzed tryptic soy broth (DTSB), an iron-depleted medium that allows robust, iron-regulated pyoverdine production by *P. aeruginosa* (71). PAO1 produced significant amounts of iron chelator in this medium, as determined by the ability of supernatants to scavenge iron from chrome azurol S (CAS) (65), while the $\Delta pvdA$ mutant produced very low levels of iron chelator (Fig. 2). Examination of the supernatants by spectroscopy, which exploits the fluorescent properties of pyoverdine, demonstrated that reduced CAS activity in the $\Delta pvdA$ mutant was due to reduced pyoverdine production (Fig. 2). Applying this analysis to the longitudinal CF isolates, we observed significant decreases of both CAS activity and fluorescence in the supernatants of the JSRI, DSAM, FCOR, and LNAP isolates from later stages of infection compared to the original isolate (Fig. 2A to D). Furthermore, the fluorescence in the supernatants of the most recent JSRI, DSAM, and FCOR isolates was comparable to that of the PAO1 $\Delta pvdA$ mutant, indicating a complete lack of pyoverdine production in these isolates. Moreover, while the CAS activity and fluorescence of the most recent LNAP isolate was comparable to those of our PAO1 laboratory strain, the levels were significantly reduced compared to LNAP-1 levels (Fig. 2D). Thus, pyoverdine production by this

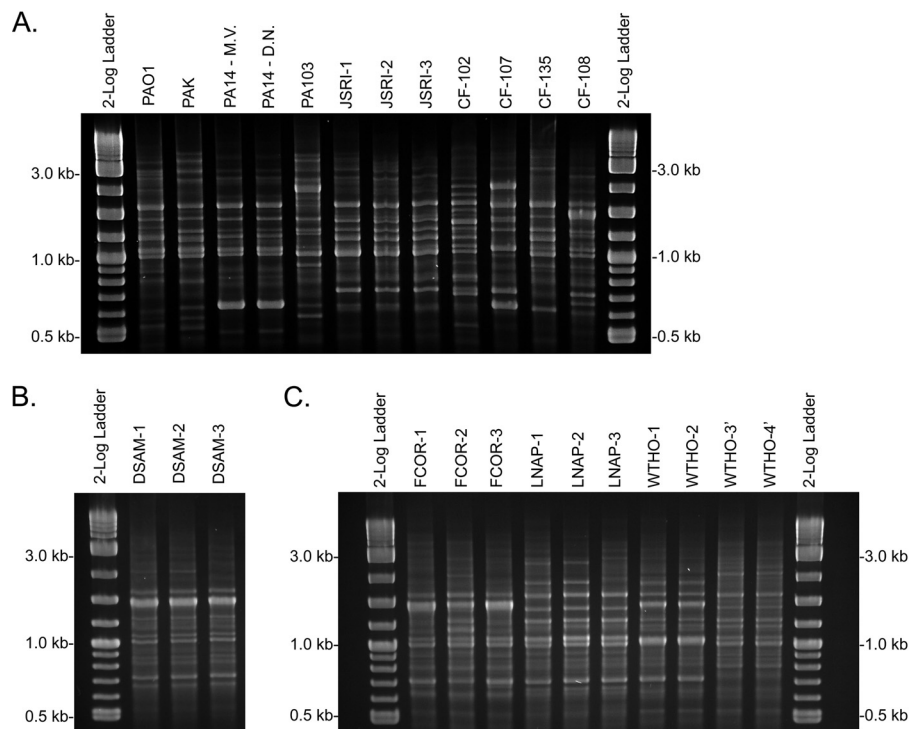


FIG 1 RAPD typing demonstrates the relatedness of longitudinal CF isolates. Genomic DNA isolated from the indicated strains was analyzed by RAPD typing as described in Materials and Methods. Strains are described in Table 1 and in Table S1 in the supplemental material.

strain also decreased during CF lung infection. No significant decrease in pyoverdine production was observed in the WTHO-2 isolate compared to the WTHO-1 isolate (Fig. 2E). However, the supernatants of the WTHO-3' isolate, which constitutes a distinct strain of *P. aeruginosa* isolated from the same patient 1 year later, demonstrated significantly less CAS activity and fluorescence than those of the WTHO-2 isolate (Fig. 2E). Decreased pyoverdine production by the JSRI, DSAM, LNAP, and WTHO strains was also observed in M9 minimal media (see Fig. S4A, B, D, and E in the supplemental material), further supporting our findings. However, FCOR-3 produced a greater level of pyoverdine in M9 than in DTSB (see Fig. S4C in the supplemental material), suggesting that the signaling pathways for pyoverdine production in this strain are distinct. Taken together, these data demonstrate that decreased production of pyoverdine is a common strategy for adapting iron uptake systems in the CF lung (Table 1).

To determine the basis of decreased pyoverdine production, we analyzed expression of genes involved in siderophore biosynthesis in the JSRI isolates grown in DTSB media. Expression of *pvdS*, encoding the sigma factor required for expression of the pyoverdine biosynthesis genes, was significantly reduced in the JSRI-1 isolate compared to PAO1 (see Fig. S5A in the supplemental material). Surprisingly, no significant reduction in *pvdS* expression was evident in the JSRI-2 isolate, even though this isolate produced significantly less pyoverdine than the JSRI-1 isolate in this medium. Thus, it is likely that mutations in the pyoverdine biosynthesis genes themselves contributed to decreased pyoverdine production in this strain. Previously, induction of pyochelin biosynthesis was observed upon loss of pyoverdine biosynthesis in *P. aeruginosa* (72). However, no significant induction of the *pchE* gene for pyochelin production was observed in either the $\Delta pvdA$

mutant or the JSRI-2 isolate upon growth in DTSB media, with or without iron supplementation (see Fig. S5B in the supplemental material). Moreover, based on the finding that *pchE* expression was consistent in the JSRI isolates, it is likely that this strain retained the ability to make pyochelin.

PQS production decreased in the JSRI strain during CF lung infection. PQS is predicted to be involved in iron acquisition by strain PAO1 through its abilities to mediate lysis of other bacteria and chelate ferric iron (57, 58). To determine if this strategy was retained by the JSRI strain during CF lung infection, we used thin-layer chromatography to analyze PQS production in high-iron and low-iron DTSB media. As expected, PAO1 produced PQS regardless of the presence or absence of iron supplementation, while no PQS was detected in the supernatant of $\Delta pqsA$, an isogenic mutant, for the first step in AQ synthesis (Fig. 3). Strikingly, JSRI-1 produced levels of PQS that were equivalent to those produced by PAO1, while AQ production was completely absent in the JSRI-2 isolate (Fig. 3). This reduction in AQ production is not likely due to decreased production of pyoverdine, as the PAO1 $\Delta pvdA$ mutant made levels of AQ that were similar to those made by wild-type PAO1 (Fig. 3). In agreement with earlier studies (62), PQS production was not increased by iron depletion, as shown by comparisons of extracts from high-iron and low-iron cultures (Fig. 3). However, our analysis showed that a distinct AQ species was produced at higher concentrations under low-iron conditions (Fig. 3). Moreover, this species was more abundant in the low-iron culture supernatants of the JSRI-1 isolate than in those of PAO1 or the $\Delta pvdA$ mutant (Fig. 3A). Analysis of the expression of *pqsA*, which encodes the first step in AQ biosynthesis from anthranilate, demonstrated that its expression was significantly decreased in JSRI-2 compared to JSRI-1 (see Fig. S5C in the

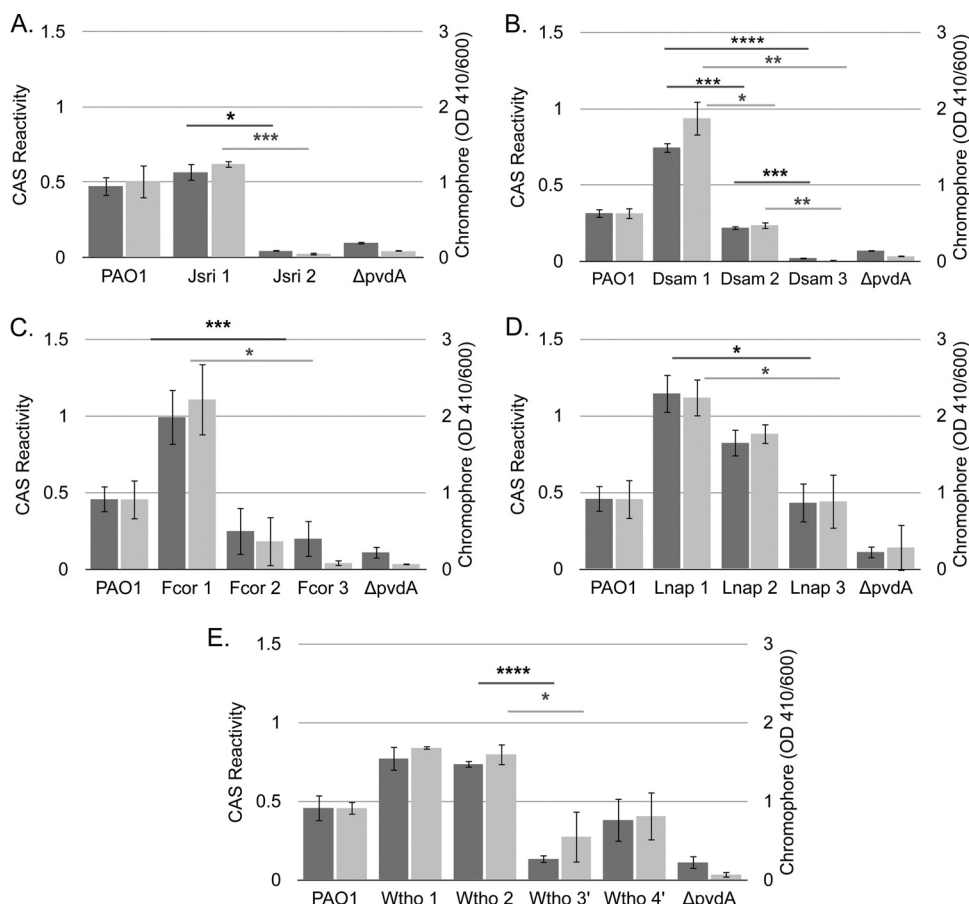


FIG 2 Identification of natural pyoverdine mutants isolated longitudinally from CF patients. The JSRI (A), DSAM (B), FCOR (C), LNAPE (D), and WTHO (E) strains were grown in DTSB media without iron supplementation at 37°C for 18 h. Cells were then harvested and supernatants analyzed for CAS reactivity (dark gray bars) and the presence of the pyoverdine chromophore (light gray bars) as described in Materials and Methods. PAO1 and $\Delta pvdA$ mutant values were also obtained in each experiment for comparison. Error bars show the standard deviations of the results of three independent experiments. Asterisks indicate the following *P* values as determined by a two-tailed Student's *t* test: *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.005; ****, *P* < 0.001. OD, optical density.

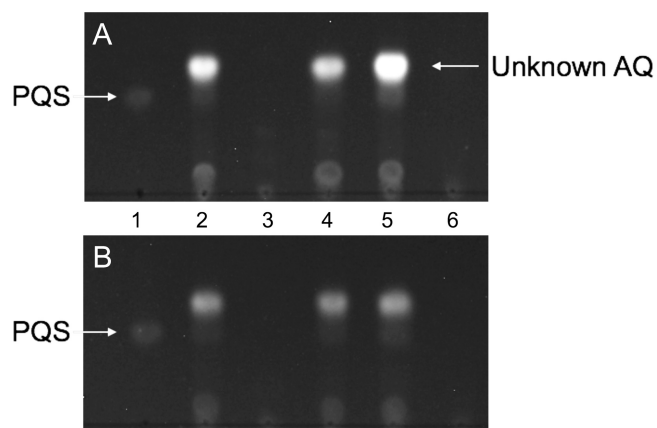


FIG 3 PQS production is eliminated in the JSRI-2 isolate. Strains were grown at 37°C for 18 h in DTSB media without (A) or with (B) supplementation of 100 μ M $FeCl_3$. Cells were harvested and supernatants were analyzed by TLC for PQS production as described in Materials and Methods. Arrows indicate the migration of PQS and an unknown iron-regulated AQ(s). Lane 1, PQS standard. Lane 2, PAO1. Lane 3, $\Delta pqsA$. Lane 4, $\Delta pvdA$. Lane 5, JSRI-1. Lane 6, JSRI-2.

supplemental material). Interestingly, we also observed some repression of *pqsA* expression by iron, although this repression was not statistically significant in any of the strains tested (see Fig. S5C). Overall, these experiments demonstrate that the ability of the JSRI strain to acquire iron via PQS was reduced during chronic CF lung infection.

Iron-regulated expression of *antA* via the PrrF sRNAs was retained by the JSRI strain during CF lung infection. The PrrF sRNAs were previously shown to be required for PQS production in the PAO1 laboratory strain (62). Thus, it was possible that the loss of PQS production in the JSRI-2 isolate was driven by decreased *prrF* expression. We therefore analyzed expression of the PrrF sRNAs by qPCR using the primers and probes shown in Fig. 4A (51). Relative to PAO1, no appreciable differences between the PAO1 $\Delta pvdA$ mutant and the JSRI isolates in *prrF* expression during growth in low-iron medium were observed (Fig. 4B). However, the JSRI-1 isolate expressed levels of the PrrF sRNAs that were nearly 10-fold higher than those of any of the other strains grown in high-iron medium (Fig. 4B). Iron repression of the PrrF sRNAs was restored in the JSRI-2 isolate (Fig. 4B and C), indicating that iron regulation of *prrF* expression became more robust in the JSRI strain during chronic CF lung infection. In contrast, no

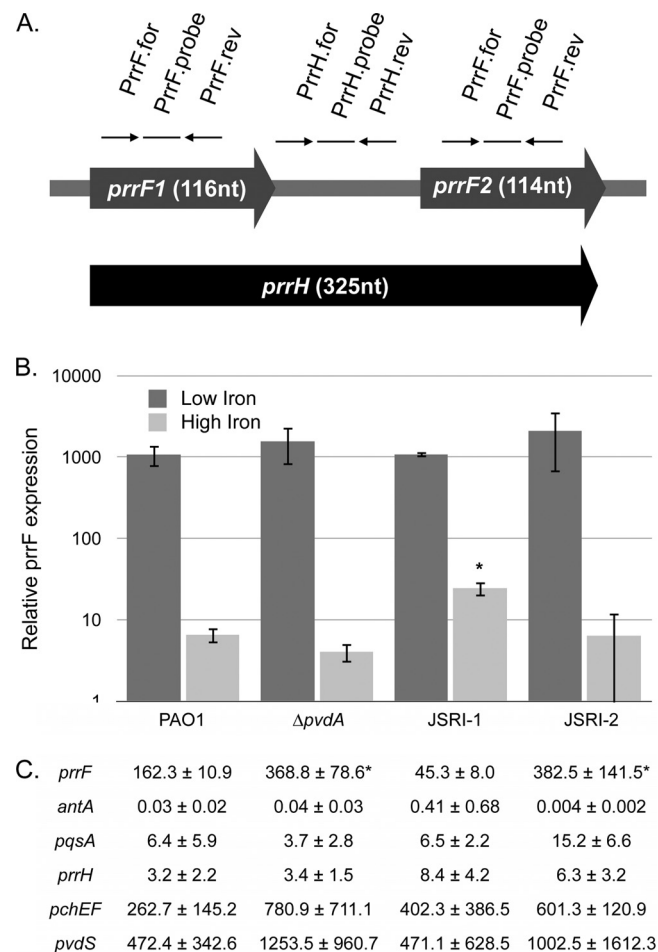


FIG 4 PrrF regulation is enhanced in the JSRI-2 isolate. (A) Map of the *prfF* locus, showing the approximate binding sites of the primers and probes used for qPCR analysis of the PrrF and PrrH sRNAs. Primers PrrF.for and PrrF.rev were used to detect all three sRNAs. Primers PrrH.for and PrrH.rev with the PrrH.probe were used to specifically detect the PrrH RNA. (B) RNA was isolated from the indicated strains grown for 18 h at 37°C in DTSE, with and without iron supplementation as indicated, and was used for qPCR analysis of the PrrF sRNAs as described in Materials and Methods. Error bars show the standard deviations of the results of three independent experiments. The asterisk (*) indicates $P < 0.05$ as determined by Student's t test for comparisons of data for the JSRI-1 strain to data for strain PAO1 grown in high-iron DTSE. (C) Iron repression of *prfF*, *prrH*, *antA*, *pvdS*, *pchE*, and *pqsA* was determined by $\Delta\Delta C_T$ analysis as described in Materials and Methods. Asterisks (*) indicate $P < 0.05$ as determined by Student's t test for comparisons of data for the JSRI-2 isolate to data for strain JSRI-1 or for comparisons of the $\Delta pvdA$ mutant to strain PAO1.

significant changes in iron-regulated *prrH* expression were observed in the PAO1 $\Delta pvdA$ mutant or in either of the JSRI isolates (Fig. 5C; see also Fig. S5E in the supplemental material).

We next determined if regulation of *antA* by the PrrF sRNAs similarly evolved in the JSRI isolates during the course of chronic CF lung infection. Iron supplementation resulted in a substantial increase in *antA* expression in both the PAO1 wild-type strain and the PAO1 $\Delta pvdA$ mutant (Fig. 4C; see also Fig. S5D in the supplemental material). Iron induction of *antA* was also observed in the JSRI-1 isolate, although less consistently than in PAO1, while iron strongly induced *antA* expression in the JSRI-2 isolate (Fig. 4C). Furthermore, increased iron induction of *antA* in the JSRI-2 iso-

late correlated with the increased repression of the PrrF sRNAs by iron in this later isolate compared to JSRI-1 (Fig. 4C). Thus, loss of PQS production in the JSRI-2 isolate is not correlated with decreased PrrF expression or regulation of the genes for anthranilate degradation. Instead, decreased *pqsA* expression in the JSRI-2 isolate compared to JSRI-1 (see Fig. S5C in the supplemental material) is likely the source of decreased PQS production. Notably, iron repression of the *pchE* and *pvdS* genes also appeared to be greater in the JSRI-2 isolate, although these changes were insignificant due to substantial experimental variation in these isolates (Fig. 4C). Overall, our data suggest that the JSRI strain became more responsive to changes in iron levels, particularly with regard to expression of the PrrF sRNAs, likely contributing to this strain's ability to adapt to the loss of pyoverdine-mediated iron uptake.

Heme acquisition was upregulated in the JSRI strain during CF lung infection. The data above indicate that loss of pyoverdine production by the JSRI strain is associated with evolution of iron regulatory pathways, which presumably function to maintain iron homeostasis in the absence of this high-affinity iron acquisition system. The heme and ferrous iron uptake systems of *P. aeruginosa* strains inhabiting the lungs of CF patients are highly expressed (33, 35). Therefore, we hypothesized that, in conjunction with reduced pyoverdine production, the JSRI strain would have up-regulated expression of heme and other iron acquisition systems. To test the ability of the PAO1 $\Delta pvdA$ mutant and JSRI isolates to use heme, we analyzed the heme metabolomes of these strains by liquid chromatography-tandem mass spectrometry (LC-MS/MS). LC prior to MS/MS fragmentation allows differentiation of the biliverdin (BVIX) isomers as a function of time—BVIX α is the product of BphO cleavage, and BVIX δ and BVIX β are the products of HemO (see Scheme S1 in the supplemental material). Furthermore, by providing cells with ^{13}C -labeled heme as an iron source, MS/MS fragmentation can detect changes in the mass-to-charge (m/z) ratio of biliverdin resulting from degradation of exogenous (^{13}C]BVIX) versus endogenous (^{12}C]BVIX) heme. While this analysis is not quantitative, ratios of labeled and unlabeled biliverdin isomers within a single sample are indicative of how a strain metabolizes heme. For example, this analysis previously showed that deletion of *phuS* in strain PAO1 results in a high ratio of ^{13}C]BVIX α , demonstrating increased dependence on BphO for heme acquisition in this mutant (44, 66). From these data, we concluded that PhuS regulates the metabolic flux of exogenous heme through the iron-regulated HemO heme oxygenase.

In agreement with previous analysis (44), growth of PAO1 under low-heme (0.5 μM) conditions resulted in BphO-mediated turnover of endogenous heme, while turnover of exogenous heme by HemO was minimal (Fig. 5). In contrast, higher concentrations (5 μM) of heme altered the ratio of BVIX derived from endogenous and exogenous heme sources, with the majority of the BVIX derived from extracellularly provided heme through the action of HemO (see Fig. S6 in the supplemental material). Similarly to strain PAO1, the PAO1 $\Delta pvdA$ mutant was not able to efficiently use heme as an iron source when lower levels (0.5 μM) of exogenous heme were provided (Fig. 5, right panels). However, the lack of intracellular heme turnover by BphO in the $\Delta pvdA$ mutant compared to PAO1 (Fig. 5) suggested that heme biosynthesis might be repressed in the absence of pyoverdine-mediated iron uptake due to iron starvation. Also in contrast to strain PAO1, supplementation of the PAO1 $\Delta pvdA$ mutant cultures with higher

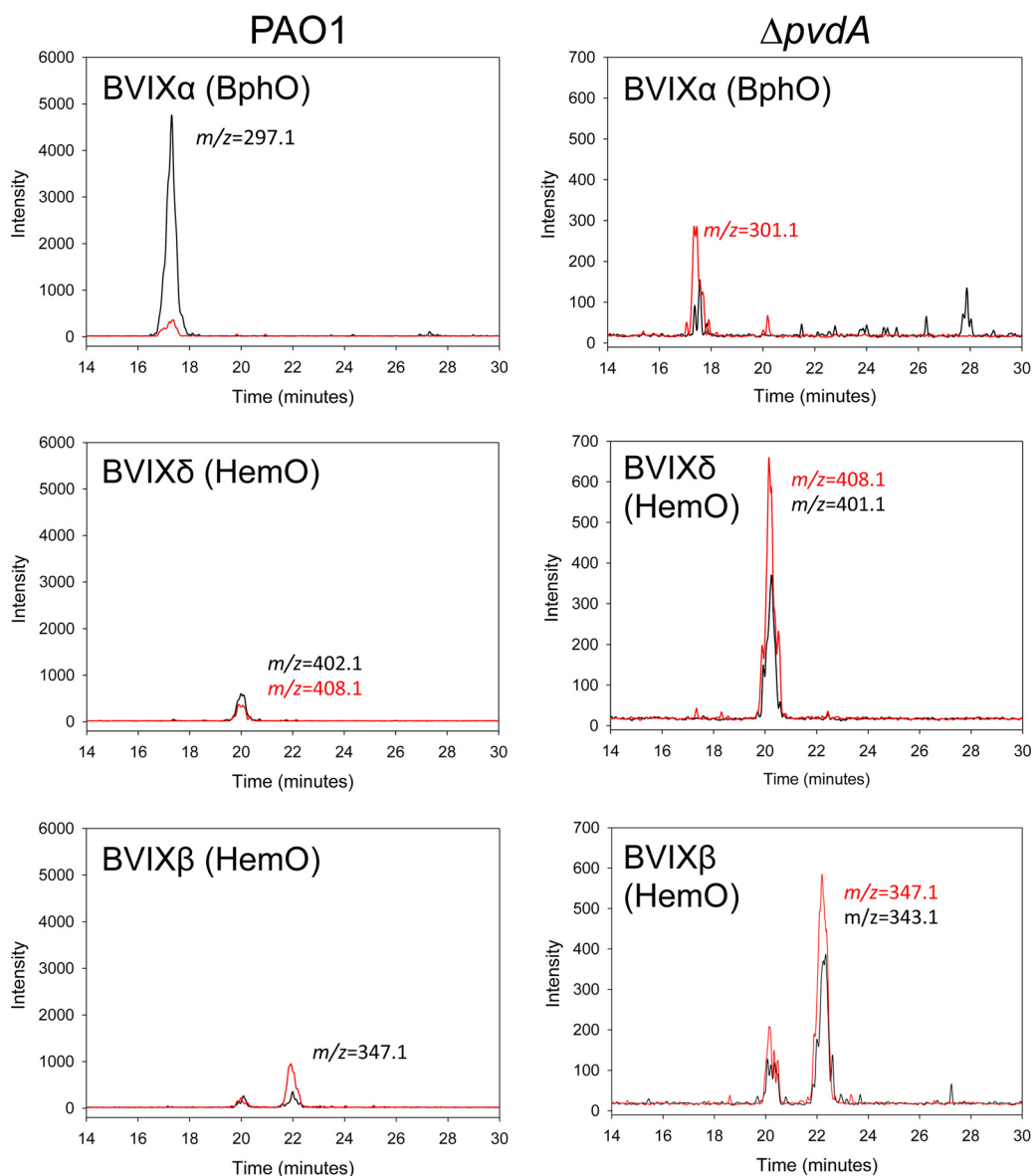


FIG 5 Intracellular heme homeostasis is disrupted in PAO1 upon the loss of pyoverdine biosynthesis. ^{13}C (red line) and ^{12}C (black) biliverdin (BVIX) was extracted from the supernatants of the indicated strains grown in M9 media with $0.5\ \mu\text{M}$ [^{13}C] heme and was subsequently analyzed by LC-MS/MS as described in Materials and Methods with multiple-reaction monitoring.

concentrations of heme ($5\ \mu\text{M}$) resulted in heme turnover by both the HemO and BphO heme oxygenases (see Fig. S6), suggesting that an uncoupling of the PhuS-HemO-driven metabolic flux of heme in the absence of pyoverdine-mediated iron uptake had occurred. These data demonstrate that heme metabolism in PAO1 is altered when pyoverdine-mediated iron acquisition is disrupted.

Strikingly, analysis of the JSRI-1 isolate revealed degradation of exogenous heme by both HemO and BphO, even at low ($0.5\ \mu\text{M}$) heme concentrations (Fig. 6, left panels), suggesting that PhuS-dependent modulation of heme flux by this isolate was completely disrupted. Similarly to the PAO1 $\Delta pvdA$ mutant, the JSRI-1 isolate also showed very little turnover of endogenous heme (Fig. 6). In contrast, the JSRI-2 isolate grown in the presence of $0.5\ \mu\text{M}$ heme degrades exogenous heme primarily through the action of HemO (see Fig. S6 in the supplemental material), demonstrating that the

JSRI strain evolved to use heme efficiently in the CF lung. Further, these data suggest that BphO-mediated degradation of exogenous heme does not allow for efficient utilization of extracellular heme as an iron source.

To further test the idea that the JSRI-2 strain evolved to use heme more efficiently as an iron source in the CF lung, we monitored expression of *phuS*, *hemO*, *bphO*, and *phuR* in each of the strains grown in the presence of low ($0.5\ \mu\text{M}$) or high ($5\ \mu\text{M}$) heme concentrations. These analyses showed increased expression levels of both *phuS* and *hemO* in the PAO1 $\Delta pvdA$ mutant, regardless of the heme concentrations (Table 2), presumably in response to iron deprivation due to the loss of pyoverdine. In contrast, expression of *phuS* and *hemO* was decreased in the JSRI-1 isolate compared to strain PAO1 in $0.5\ \mu\text{M}$ heme (Table 2), likely resulting in the observed “shunting” of extracellular heme

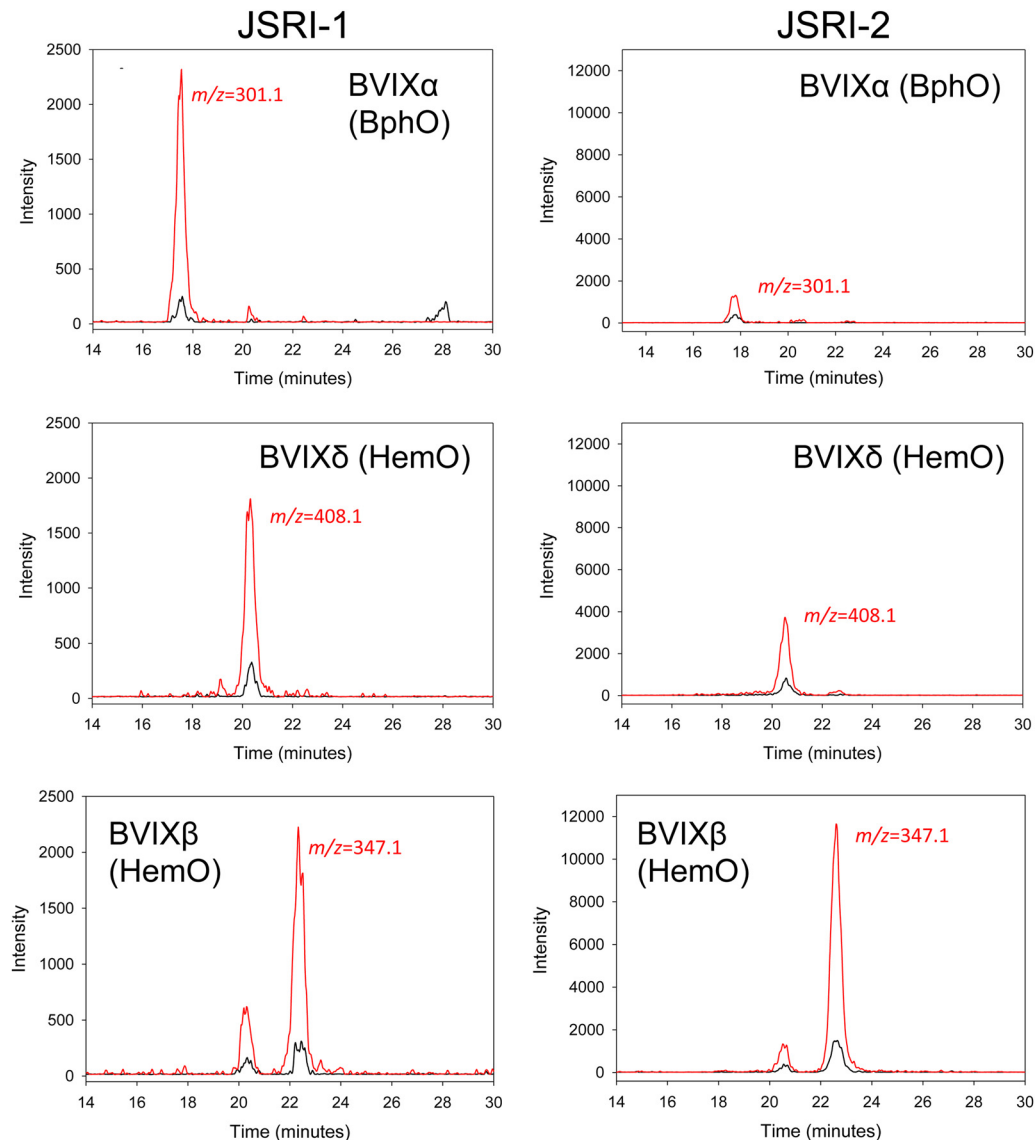


FIG 6 Heme acquisition became more efficient in the JSRI strain during CF lung infection. ^{13}C (red line) and ^{12}C (black) biliverdin (BVIX) was extracted from the supernatants of the indicated strains grown in M9 media with $0.5\ \mu\text{M}$ [^{13}C]heme and was subsequently analyzed by LC-MS/MS as described in Materials and Methods with multiple-reaction monitoring.

through BphO (Fig. 6). Combined with our analysis of the heme metabolites presented above, these data indicate that any perturbations in PhuS levels can disrupt the flux of extracellular heme through the cell. Expression of *phuR* was also decreased in the JSRI-1 isolate compared to strain PAO1 (Table 2), which may also have contributed to inefficient utilization of heme as an iron source in the earlier isolate. As expected, the expression levels of *phuS*, *hemO*, and *phuR* in the JSRI-2 isolate had returned to PAO1 levels (Table 2), resulting in enhanced heme flux through HemO in this isolate. Thus, the JSRI-1 and JSRI-2 isolates represent distinct stages in the adaptation of the iron acquisition and regulatory networks of this strain, which resulted in more-efficient heme utilization by the JSRI-2 isolate.

***P. aeruginosa* expresses heme oxygenase and the PrrF sRNAs in the CF lung.** Our analyses of the JSRI isolates presented above suggest that *P. aeruginosa* altered its iron acquisition and regula-

tory pathways to adapt to the unique environment of the CF lung, specifically with regard to PrrF regulation and heme acquisition. While these analyses are highly suggestive of the evolutionary pressures that exist in the CF lung, they do not confirm that these systems are in fact expressed and utilized by *P. aeruginosa* during infection. The *phuR* and *phuS* genes that encode an outer membrane receptor for heme and an intracellular heme chaperone, respectively, are highly represented in RNA isolated from preserved *P. aeruginosa* sputum samples from CF patients (33). Here, we extended this analysis to the expression of *hemO* and *bphO*. Transcripts of these genes were detected in all sputum samples tested (Fig. 7A). These included four sputum samples from three CF patients, as well as three sputum samples from non-CF bronchiectasis patients, who also commonly suffer from chronic *P. aeruginosa* infections (73). We also tested for expression of the PrrF and PrrH RNAs in 10 distinct CF patients (Fig. 7B). These

TABLE 2 Expression analysis of heme metabolism genes

Gene	Strain	Fold change compared to PAO1 ^a	
		0.5 μ M heme	5 μ M heme
<i>phuS</i>	Δ <i>pvdA</i>	3.55 \pm 0.49**	2.62 \pm 1.61
	JSRI-1	0.44 \pm 0.10*	0.50 \pm 0.04**
	JSRI-2	0.86 \pm 0.13	0.99 \pm 0.32
<i>hemO</i>	Δ <i>pvdA</i>	1.62 \pm 0.17**	5.21 \pm 0.16***
	JSRI-1	0.24 \pm 0.02***	0.90 \pm 0.05
	JSRI-2	0.60 \pm 0.04***	0.76 \pm 0.04
<i>bphO</i>	Δ <i>pvdA</i>	1.18 \pm 0.14	7.98 \pm 0.50***
	JSRI-1	5.84 \pm 1.28**	1.60 \pm 0.08**
	JSRI-2	4.05 \pm 0.83**	2.89 \pm 0.50**
<i>phuR</i>	Δ <i>pvdA</i>	0.78 \pm 0.063	0.52 \pm 1.61
	JSRI-1	0.17 \pm 0.062**	0.74 \pm 0.043**
	JSRI-2	0.49 \pm 0.074	0.59 \pm 0.098**

^a Fold change determined by qPCR of the indicated strains grown in M9 media as described in the Materials and Methods. Standard deviations are of three biological replicates. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$ (as determined by a two-tailed Student's *t* test for comparisons of the data for each strain to data for PAO1 grown under the same conditions).

analyses showed that the PrrF sRNAs were expressed at levels that were extremely high (anywhere from 10- to 100-fold higher than the levels seen with the reference genes) in these patients and much higher than those seen with any other genes tested by this technique thus far. Expression of the PrrF sRNAs was also very high in strain PAO1 grown in low-iron medium *in vitro* (575.2-fold over expression of the reference genes in King's B medium [33]) but was significantly repressed by iron (0.509-fold change in comparison to the reference genes when supplemented with 100 μ g/ml ferric chloride). These data suggest that the PrrF sRNAs are very highly expressed in environments where iron is limiting, including the CF lung. Expression of the PrrH RNA was not nearly as high as that seen with PrrF in CF sputum or *in vitro* (about 1/100th of PrrF expression) but was still detected in every sample tested and was equivalent to what is observed in strain PAO1 grown under low-iron conditions (Fig. 7B). These data show that the PrrF and PrrH sRNAs, as well as the *hemO*- and *bphO*-encoded heme oxygenases, are expressed by *P. aeruginosa* in the CF lung environment. Combined with our analysis of the longitudinally isolated JSRI strain, these data argue that PrrF-mediated iron regulation and heme acquisition are likely important contributors to survival in, and adaptation to, the CF lung.

DISCUSSION

During chronic CF lung infections, *P. aeruginosa* undergoes significant changes as it adapts to the host lung, the physiology of which is also constantly changing due to lung tissue deterioration (9). In contrast to studies showing the genetic evolution of *P. aeruginosa* during CF lung infection (34, 74–78), we sought to understand how this evolution drives changes in iron homeostasis, which is a key facet of *P. aeruginosa* pathogenesis (17–22). Growing evidence suggests that *P. aeruginosa* becomes less dependent on pyoverdine as a means for iron acquisition during CF lung infection, most likely due to increased availability of heme and ferrous iron as lung function declines and oxygen levels decrease (32, 33, 35). Here, we analyzed numerous longitudinally isolated

strains of *P. aeruginosa* from multiple CF patients and show that the majority reduced their capacity to produce pyoverdine during lung infection. Although it has low resolution compared to genome sequencing, RAPD typing of these isolates provided a quick and reproducible technique to determine the clonality of several of these strains despite genomic changes leading to mucoidy and loss of pyoverdine (59). Further analysis of the JSRI strain showed that its iron regulatory and heme acquisition pathways were altered extensively in the CF lung as pyoverdine production was reduced. In particular, analysis of biliverdin production by this strain provided snapshots of its heme metabolic capacity at two distinct points during CF lung infection, revealing a novel mechanism by which *P. aeruginosa* can adapt to preferentially use heme as an iron source. We further validated our findings with *in vivo* expression data from CF patients, which showed that the heme metabolism and iron regulatory pathways examined in this study are also expressed by *P. aeruginosa* strains in the CF lung. As such, this work provides a critical foundation for development of novel and appropriate drug targets, demonstrating that both iron regulation and heme acquisition are viable foci for novel antimicrobial development.

One of the most exciting aspects of this study was the ability to track CF-dependent changes of iron and heme acquisition in individual strains as a function of time. Additionally, analysis of heme metabolism by mass spectrometry and qPCR shows that the JSRI strain adapted its heme regulatory networks in conjunction with reduced production of pyoverdine. Specifically, the JSRI-2 isolate efficiently uses heme to meet its iron needs, while the

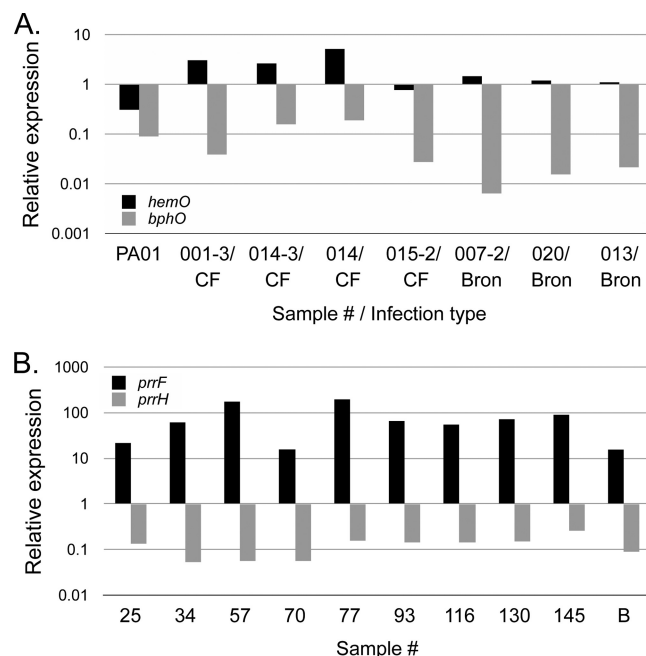


FIG 7 *prrF* and *hemO* are expressed by *P. aeruginosa* infecting CF and bronchiectasis patients. Sputum from CF and bronchiectasis (Bron) patients chronically infected with *P. aeruginosa* was collected and preserved with RNALater. RNA was subsequently isolated and analyzed for expression of *hemO* and *bphO* (A) or *prrF* and *prrH* (B), and relative values were normalized to *clpX* and *oprL* as described in Materials and Methods. Samples 014-3 and 014 (A) were isolated at separate times (approximately 3 months apart) from the same CF patient. Relative levels of expression of *hemO* and *bphO* in PAO1 were determined *in vitro* in King's B medium.

JSRI-1 isolate appears to be “mid-evolution” from pyoverdine-mediated iron acquisition to efficient heme utilization. Our studies also showed that extracellular heme degradation by BphO, which is normally involved in endogenous heme turnover, is an indicator of iron starvation and does not appear to contribute to efficient heme utilization. Recent work shows that heme and ferrous iron uptake systems are consistently expressed in the CF lung (33) and that ferrous iron becomes more abundant in the CF lung as disease progresses (35). The metabolomics data in this study provide a novel insight into how *P. aeruginosa* adapts to these changes, indicating that HemO is the driver of enhanced heme acquisition by the JSRI strain. Moreover, our *in vivo* data demonstrate that HemO is expressed by *P. aeruginosa* during CF lung infection as well as in bronchiectasis patients. Thus, the shift in dependence of the JSRI strain from siderophore-dependent iron uptake to heme acquisition is likely dependent on this critical mediator of heme metabolism.

In light of the heme metabolomics data, it is somewhat surprising that no changes in expression of the heme-responsive PrrH RNA were observed between the JSRI-1 and JSRI-2 isolates. One would expect that changes in heme acquisition and metabolism would correlate with changes in heme-regulated genes. However, the current study focused on iron-regulated expression of the PrrF and PrrH sRNAs. Perhaps future exploration of heme-regulated PrrH expression in the JSRI and other CF isolates will reveal new clinical significance for this unique regulatory RNA. In contrast to PrrH results, iron-regulated expression of the PrrF sRNAs was enhanced in the JSRI-2 isolate compared to JSRI-1, suggesting the presence of a mechanism by which this strain may have become more sensitive to fluctuations in iron availability. Moreover, the high levels of PrrF that were detected in sputa from multiple CF patients strongly suggest that this sRNA plays a central regulatory role throughout CF lung infections. Furthermore, our data show that the ratio of PrrH sRNA to PrrF sRNA is retained *in vivo*, suggesting that regulation by this unique heme-responsive sRNA is also important for chronic CF lung infection by *P. aeruginosa*.

Anthranilate is the precursor for the PQS quorum-sensing molecule, and PrrF repression of the anthranilate degradation genes (*antABC*) was previously shown to be required for PQS production in low-iron environments (62). In spite of the finding that this regulatory pathway appears to be conserved in the JSRI strain, PQS production was eliminated in the JSRI strain at later stages of CF lung infection (Fig. 3). Our data suggest that this loss is due in part to reduced *pqsA* expression, although further studies will be needed to determine the underlying mechanism of decreased PQS production in the JSRI strain. An interesting issue that our study raised is whether or not PQS production is decreased in other CF isolates during chronic lung infection. A previous study detected PQS in sputum and bronchoalveolar lavage fluid of CF patients, and the authors further showed that PQS was made by most (9/10) *P. aeruginosa* CF isolates tested in that study (67). However, the age of the patients from which the strains were isolated in that study was not included, so it is unclear if PQS production by the strains changed as a function of time. Studies on acyl-homoserine lactone quorum-sensing signals in CF patients have shown that, while these compounds are identified in the sputa of CF patients (79), their production by individual strains decreases over time due to genetic mutation (12, 80). More-extensive studies of PQS production in longitudinal isolates of *P. aeruginosa* are clearly needed to determine if production of the

PQS quorum-sensing molecule is similarly lost during chronic CF lung infection.

Overall, our report provides a much-needed appraisal of how naturally occurring *P. aeruginosa* pyoverdine mutants acquire iron during chronic CF lung infections, with longitudinal isolates yielding a powerful system for tracking evolution of these strains. Future expansion of these studies to a larger collection of longitudinal CF isolates is likely to reveal many more novel features of evolution of *P. aeruginosa* iron uptake systems in the CF lung.

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